

Applicants: Peter S. Linsley et al.
U.S. Serial No.: 09/454,651
Filed: December 6, 1999
Page 2

In the Title

In accordance with 37 C.F.R. §§1.121(b)(1)(i)-(ii), please delete the title on page 1, beginning

“CTL4 MOLECULES AND IL4-BINDING MOLECULES AND USES THEREOF”

and replace it with the following rewritten title:

*Change to
Title*
-- METHODS FOR INHIBITING INTERACTIONS BETWEEN CTLA4-POSITIVE T
CELLS AND B7 POSITIVE CELLS --

In the Specification:

Please delete the paragraph at page 1, line 6, beginning, “This application is a continuation-in-part of U.S. Serial No. 08/008,898, filed January 22, 1993, which is a continuation-in-part of U.S. Serial No. 723,617, filed July 27, 1991, now abandoned, the contents of all of which are incorporated by reference into the present application” and replace it with the following rewritten paragraph:

D1 --This application is a divisional application of U.S. Serial No. 08/228,208, filed April 15, 1994, now U.S. Patent No. 6,090,914, which is a continuation-in-part of U.S. Serial No. 08/008,898, filed January 22, 1993, now U.S. Patent No. 5,776,197, which is a continuation-in-part of U.S. Serial No. 723,617, filed July 27, 1991, now abandoned, the contents of all of which are incorporated by reference in their entirety into this application. -

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At page 21, line 5, please insert:

D2 -- The predicted amino acid sequence for amino acids 1-216 of the B7 antigen (SEQ ID NO: 23), isolated by Freeman et al., (*Supra*) is:

Applicants: Peter S. Linsley et al.
U.S. Serial No.: 09/454,651
Filed: December 6, 1999
Page 3

Gly Leu Ser His Phe Cys Ser Gly Val Ile His Val Thr Lys Glu Val

1 5 10 15

Lys Glu Val Ala Thr Leu Ser Cys Gly His Asn Val Ser Val Glu Glu

20 25 30

Leu Ala Gln Thr Arg Ile Tyr Trp Gln Lys Glu Lys Lys Met Val Leu

35 40 45

Thr Met Met Ser Gly Asp Met Asn Ile Trp Pro Glu Tyr Lys Asn Arg

50 55 60

Thr Ile Phe Asp Ile Thr Asn Asn Leu Ser Ile Val Ile Leu Ala Leu

65 70 75 80

Arg Pro Ser Asp Glu Gly Thr Tyr Glu Cys Val Val Leu Lys Tyr Glu

85 90 95

Lys Asp Ala Phe Lys Arg Glu His Leu Ala Glu Val Thr Leu Ser Val

100 105 110

Lys Ala Asp Phe Pro Thr Pro Ser Ile Ser Asp Phe Glu Ile Pro Thr

115 120 125

Ser Asn Ile Arg Arg Ile Ile Cys Ser Thr Ser Gly Gly Phe Pro Glu

130 135 140

Pro His Leu Ser Trp Leu Glu Asn Gly Glu Glu Leu Asn Ala Ile Asn

D2

D2

Please delete the paragraph at page 21, line18, beginning, "Because the expression of CTLA4 receptor protein in human lymphoid cells has not been previously reported, it was necessary to locate a source of CTLA4 mRNA. PCR cDNA made from the total cellular RNA of several human leukemia cell lines was screened, using as primers, oligonucleotides from the published sequence of the CTLA4 gene (Dariavach et al., supra). Of the cDNA tested, H38 cells (an HTLV II-associated leukemia line) provided the best yield of PCR products having the expected size. Since a signal peptide for CTLA4 was not identified in the CTLA4 gene, the N terminus of the predicted sequence of CTLA4 was fused to the signal peptide of oncostatin M (Malik et al., Molec. and Cell. Biol. 9:2847 (1989)) in two steps using oligonucleotides as described in the Examples, infra. The product of the PCR reaction was ligated with cDNA encoding the amino acid sequences corresponding to the hinge, CH2 and CH3 regions of Ig C 1 into an expression vector, such as CDM8 or π LN." and replace it with the following rewritten paragraph:

D3 -- Because the expression of CTLA4 receptor protein in human lymphoid cells has not been previously reported, it was necessary to locate a source of CTLA4 mRNA. PCR cDNA made from the total cellular RNA of several human leukemia cell lines was screened, using as primers, oligonucleotides from the published sequence of the CTLA4 gene (Dariavach et al., supra). Of the cDNA tested, H38 cells (an HTLV II-associated leukemia line) provided the best yield of PCR products having the expected size. Since a signal peptide for CTLA4 was not identified in the CTLA4 gene, the N terminus of the predicted sequence of CTLA4 was fused to the signal peptide of oncostatin M (Malik et al., Molec. and Cell. Biol. 9:2847 (1989)) in two steps using oligonucleotides as described in the Examples, infra. The product of the PCR reaction was ligated with cDNA encoding the amino acid sequences corresponding to the hinge, CH2 and CH3 regions of Ig C 1 into an expression vector, such as CDM8 or π LN. -

Please delete the paragraph at page 22, line 32, beginning, "The clones containing DNA encoding fusion constructs obtained as described above are then transfected into suitable host cells for expression. Depending on the host cell used, transfection is performed using standard techniques appropriate to such cells. For example, transfection into mammalian cells is accomplished using DEAE-dextran mediated transfection, CaPO₄ co-precipitation, lipofection, electroporation, or protoplast fusion, and other methods known in the art including: lysozyme fusion or erythrocyte fusion, scraping, direct uptake, osmotic or sucrose shock, direct microinjection, indirect microinjection such as via erythrocyte-mediated techniques, and/or by subjecting host cells to electric currents. The above list of transfection techniques is not considered to be exhaustive, as other procedures for introducing genetic information into cells will no doubt be developed." and replace it with the following rewritten paragraph:

D4 -- The clones containing DNA encoding fusion constructs obtained as described above are then transfected into suitable host cells for expression. Depending on the host cell used, transfection is performed using standard techniques appropriate to such cells. For example,

D4
transfection into mammalian cells is accomplished using DEAE-DextranTM mediated transfection, CaPO₄ co-precipitation, lipofection, electroporation, or protoplast fusion, and other methods known in the art including: lysozyme fusion or erythrocyte fusion, scraping, direct uptake, osmotic or sucrose shock, direct microinjection, indirect microinjection such as via erythrocyte-mediated techniques, and/or by subjecting host cells to electric currents. The above list of transfection techniques is not considered to be exhaustive, as other procedures for introducing genetic information into cells will no doubt be developed. --.

Please delete the paragraph at page 33, line 26, beginning, "For example, the present invention affects the transplant antigen-specific T cells, thus inducing donor-specific and antigen-specific tolerance. The binding of CD28 by its ligand, B7/BB1 (B7), during T cell receptor engagement is critical for proper T cell signaling in some systems (M. K. Jenkins, P. S. Taylor, S. D. Norton, K. B. Urdahl, J. Immunol. 147:2461 (1991); C. H. June, J. A. Ledbetter, P. S. Linsley, C. B. Thompson, Immunol. Today 11:211 (1990); H. Reiser, G. J. Freeman, Z. Razi-Wolf, C. D. Gimmi, B. Benacerraf, L. M. Nadler, Proc. Natl. Acad. Sci. U.S.A. 89:271 (1992); N. K. Damie, K. Klussman, P. S. Linsley, A. Aruffo, J. Immunol. 148:1985 (1992))." and replace it with the following rewritten paragraph:

D5
-- For example, the present invention affects the transplant antigen-specific T cells, thus inducing donor-specific and antigen-specific tolerance. The binding of CD28 by its ligand, B7/BB1 (B7), during T cell receptor engagement is critical for proper T cell signaling in some systems (M. K. Jenkins, P. S. Taylor, S. D. Norton, K. B. Urdahl, J. Immunol. 147:2461 (1991); C. H. June, J. A. Ledbetter, P. S. Linsley, C. B. Thompson, Immunol. Today 11:211 (1990); H. Reiser, G. J. Freeman, Z. Razi-Wolf, C. D. Gimmi, B. Benacerraf, L. M. Nadler, Proc. Natl. Acad. Sci. U.S.A. 89:271 (1992); N. K. Damle, K. Klussman, P. S. Linsley, A. Aruffo, J. Immunol. 148:1985 (1992)). --.

Please delete the paragraph at page 37, line 16, beginning, "Cell Culture and Transfections. COS (monkey kidney cells) were transfected with expression plasmids expressing CD28

and B7 using a modification of the protocol of Seed and Aruffo (Proc. Natl. Acad. Sci. 84:3365 (1987)), incorporated by reference herein. Cells were seeded at 10^6 per 10 cm diameter culture dish 18-24 h before transfection. Plasmid DNA was added (approximately 15 μ g/dish) in a volume of 5 mls of serum-free DMEM containing 0.1 mM chloroquine and 600 μ g/ml DEAE Dextran, and cells were incubated for 3-3.5 h at 37°C. Transfected cells were then briefly treated (approximately 2 min) with 10% dimethyl sulfoxide in PBS and incubated at 37°C for 16-24 h in DMEM containing 10% FCS. At 24 h after transfection, culture medium was removed and replaced with serum-free DMEM (6 ml/dish). Incubation was continued for 3 days at 37°C, at which time the spent medium was collected and fresh serum-free medium was added. After an additional 3 days at 37°C, the spent medium was again collected and cells were discarded.” and replace it with the following rewritten paragraph:

D6 -- Cell Culture and Transfections. COS (monkey kidney cells) were transfected with expression plasmids expressing CD28 and B7 using a modification of the protocol of Seed and Aruffo (Proc. Natl. Acad. Sci. 84:3365 (1987)), incorporated by reference herein. Cells were seeded at 10^6 per 10 cm diameter culture dish 18-24 h before transfection. Plasmid DNA was added (approximately 15 μ g/dish) in a volume of 5 mls of serum-free DMEMTM containing 0.1 mM chloroquine and 600 μ g/ml DEAE DextranTM, and cells were incubated for 3-3.5 h at 37°C. Transfected cells were then briefly treated (approximately 2 min) with 10% dimethyl sulfoxide in PBS and incubated at 37°C for 16-24 h in DMEMTM containing 10% FCS. At 24 h after transfection, culture medium was removed and replaced with serum-free DMEMTM (6 ml/dish). Incubation was continued for 3 days at 37°C, at which time the spent medium was collected and fresh serum-free medium was added. After an additional 3 days at 37°C, the spent medium was again collected and cells were discarded. -

Please delete the paragraph at page 37, line 33, beginning, “CHO cells expressing CD28, CD5 or B7 were isolated as described by Linsley et al., (1991) supra, as follows: Briefly,

stable transfectants expressing CD28, CD5, or B7, were isolated following cotransfection of dihydrofolate reductase-deficient Chinese hamster ovary (dhfr⁻ CHO) cells with a mixture of the appropriate expression plasmid and the selectable marker, pSV2dhfr (Linsley et al., Proc. Natl. Acad. Sci. USA 87:5031 (1990)), incorporated by reference herein. Transfectants were then grown in increasing concentrations of methotrexate to a final level of 1 μ M and were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 0.2 mM proline and 1 μ M methotrexate. CHO lines expressing high levels of CD28 (CD28⁺ CHO) or B7 (B7⁺ CHO) were isolated by multiple rounds of fluorescence-activated cell sorting (FACS^R) following indirect immunostaining with mAbs 9.3 or BB-1. Amplified CHO cells negative for surface expression of CD28 or B7 (dhfr⁺ CHO) were also isolated by FACS^R from CD28-transfected populations." and replace it with the following rewritten paragraph:

D7 -- CHO cells expressing CD28, CD5 or B7 were isolated as described by Linsley et al., (1991) supra, as follows: Briefly, stable transfectants expressing CD28, CD5, or B7, were isolated following cotransfection of dihydrofolate reductase-deficient Chinese hamster ovary (dhfr⁻ CHO) cells with a mixture of the appropriate expression plasmid and the selectable marker, pSV2dhfr (Linsley et al., Proc. Natl. Acad. Sci. USA 87:5031 (1990)), incorporated by reference herein. Transfectants were then grown in increasing concentrations of methotrexate to a final level of 1 μ M and were maintained in DMEMTM supplemented with 10% fetal bovine serum (FBS), 0.2 mM proline and 1 μ M methotrexate. CHO lines expressing high levels of CD28 (CD28⁺ CHO) or B7 (B7⁺ CHO) were isolated by multiple rounds of fluorescence-activated cell sorting (FACS^R) following indirect immunostaining with mAbs 9.3 or BB-1. Amplified CHO cells negative for surface expression of CD28 or B7 (dhfr⁺ CHO) were also isolated by FACS^R from CD28-transfected populations. --

Please delete the paragraph at page 38, line 15, beginning, "Immunostaining and FACS^R Analysis". Transfected CHO or COS cells or activated T cells were analyzed by indirect

immunostaining. Before staining, CHO cells were removed from their culture vessels by incubation in PBS containing 10 mM EDTA. Cells were first incubated with murine mAbs 9.3 (Hansen et al., Immunogenetics 10:247 (1980)) or BB-1 (Yokochi et al., J. Immunol. 128:823 (1981)), or with Ig fusion proteins (all at 10 g/ml in DMEM containing 10% FCS) for 1-2 h at 4°C. Cells were then washed, and incubated for an additional 0.5-2h at 4°C with a FITC-conjugated second step reagent (goat anti-mouse Ig serum for murine mAbs, or goat anti-human Ig C serum for fusion proteins (Tago, Inc., Burlingame, CA)). Fluorescence was analyzed on a FACS IV^R cell sorter (Becton Dickinson and CO., Mountain View, CA) equipped with a four decade logarithmic amplifier." and replace it with the following rewritten paragraph:

D8 -- Immunostaining and FACS^R Analysis. Transfected CHO or COS cells or activated T cells were analyzed by indirect immunostaining. Before staining, CHO cells were removed from their culture vessels by incubation in PBS containing 10 mM EDTA. Cells were first incubated with murine mAbs 9.3 (Hansen et al., Immunogenetics 10:247 (1980)) or BB-1 (Yokochi et al., J. Immunol. 128:823 (1981)), or with Ig fusion proteins (all at 10 g/ml in DMEMTM containing 10% FCS) for 1-2 h at 4°C. Cells were then washed, and incubated for an additional 0.5-2h at 4°C with a FITC-conjugated second step reagent (goat anti-mouse Ig serum for murine mAbs, or goat anti-human Ig C serum for fusion proteins (Tago, Inc., Burlingame, CA)). Fluorescence was analyzed on a FACS IV^R cell sorter (Becton Dickinson and CO., Mountain View, CA) equipped with a four decade logarithmic amplifier. --

Please delete the paragraph at page 40, line 29, beginning "Expression plasmids, CDM8, containing CTLA4Ig were then transfected into COS cells using DEAE/dextran transfection by modification (Linsley et al., 1991, supra) of the protocol described by Seed and Aruffo, 1987, supra." and replace it with the following rewritten paragraph:

D9

-- Expression plasmids, CDM8, containing CTLA4Ig were then transfected into COS cells using DEAE/DextranTM transfection by modification (Linsley et al., 1991, supra) of the protocol described by Seed and Aruffo, 1987, supra. --

Please delete the paragraph at page 41, line 8, beginning "A preferred stable transfectant, expressing CTLA4Ig, designated Chinese Hamster Ovary Cell Line, CTLA4Ig-24, was made by screening B7 positive CHO cell lines for B7 binding activity in the medium using immunostaining. Transfectants were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 0.2 mM proline and 1 μ M methotrexate." and replace it with the following rewritten paragraph:

D10

-- A preferred stable transfectant, expressing CTLA4Ig, designated Chinese Hamster Ovary Cell Line, CTLA4Ig-24, was made by screening B7 positive CHO cell lines for B7 binding activity in the medium using immunostaining. Transfectants were maintained in DMEMTM supplemented with 10% fetal bovine serum (FBS), 0.2 mM proline and 1 μ M methotrexate.

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Please delete the paragraph at page 42, line 5, beginning "To reconstruct DNA encoding the amino acid sequence corresponding to the full length human CTLA4 gene, cDNA encoding amino acids corresponding to a fragment of the transmembrane and cytoplasmic domains of CTLA4 was cloned by PCR and then joined with cDNA encoding amino acids corresponding to a fragment from CTLA4Ig that corresponded to the oncostatin M signal peptide fused to the N-terminus of CTLA4. Procedures for PCR, and cell culture and transfections were as described above in Example 1 using COS cells and DEAE-dextran transfection." and replace it with the following rewritten paragraph:

D11

-- To reconstruct DNA encoding the amino acid sequence corresponding to the full length human CTLA4 gene, cDNA encoding amino acids corresponding to a fragment of the transmembrane and cytoplasmic domains of CTLA4 was cloned by PCR and then joined

D11
with cDNA encoding amino acids corresponding to a fragment from CTLA4Ig that corresponded to the oncostatin M signal peptide fused to the N-terminus of CTLA4. Procedures for PCR, and cell culture and transfections were as described above in Example 1 using COS cells and DEAE-DextranTM transfection. --

Please delete the paragraph at page 44, line 1, beginning "Immunostaining and FACS^R Analysis". Prior to staining, COS or CHO cells were removed from their culture vessels by incubation in PBS containing 10 mM EDTA. Cells were first incubated with mAbs or Ig fusion proteins at 10 µg/ml in DMEM containing 10% FBS for 1-2 hr at 4° C. Cells were then washed, and incubated for an additional 0.5-2 hrs at 4° C with FITC-conjugated goat anti-mouse immunoglobulin or with FITC-conjugated goat anti-human Ig Gγ serum (both from Tago, Burlingame, CA). When binding of both mAbs and Ig fusion proteins were measured in the same experiment, FITC-conjugated anti-mouse and anti-human second step reagents were mixed together before use. Fluorescence on a total of 10,000 cells was then analyzed by FACS^R." and replace it with the following rewritten paragraph:

D12
-- Immunostaining and FACS^R Analysis. Prior to staining, COS or CHO cells were removed from their culture vessels by incubation in PBS containing 10 mM EDTA. Cells were first incubated with mAbs or Ig fusion proteins at 10 µg/ml in DMEMTM containing 10% FBS for 1-2 hr at 4°C. Cells were then washed, and incubated for an additional 0.5-2 hrs at 4°C with FITC-conjugated goat anti-mouse immunoglobulin or with FITC-conjugated goat anti-human Ig Gγ serum (both from Tago, Burlingame, CA). When binding of both mAbs and Ig fusion proteins were measured in the same experiment, FITC-conjugated anti-mouse and anti-human second step reagents were mixed together before use. Fluorescence on a total of 10,000 cells was then analyzed by FACS^R. --

Please delete the paragraph at page 44, line 14, beginning "Peripheral Blood Lymphocyte Separation and Stimulation". Peripheral blood lymphocytes (PBLs) were isolated by centrifugation through Lymphocyte Separation Medium (Litton Bionetics, Kensington,

MD). Alloreactive T cells were isolated by stimulation of PBL in a primary mixed lymphocyte reaction (MLR). PBL were cultured at 10^6 /ml irradiated (5000 rad) T51 LCL. EBV-transformed lymphoblastoid cell lines (LCL), PM (Bristol-Myers Squibb Co.) and T51 (Bristol-Myers Squibb Co.) were maintained in RPMI supplemented with 10% FBS. After 6 days, alloreactive "blasts" cells were cryopreserved. Secondary MLR were conducted by culturing thawed alloreactive blasts together with fresh irradiated T51 LCL in the presence and absence of mAbs and Ig fusion proteins. Cells were cultured in 96 well flat bottom plates (4×10^4 alloreactive blasts and 1×10^4 irradiated T51 LCL cells/well, in a volume of 0.2 ml) in RPMI containing 10% FBS. Cellular proliferation of quadruplicate cultures was measured by uptake of [3 H]-thymidine during the last 6 hours of a 2-3 day culture." and replace it with the following rewritten paragraph:

D13 -- Peripheral Blood Lymphocyte Separation and Stimulation. Peripheral blood lymphocytes (PBLs) were isolated by centrifugation through Lymphocyte Separation MediumTM (Litton Bionetics, Kensington, MD). Alloreactive T cells were isolated by stimulation of PBL in a primary mixed lymphocyte reaction (MLR). PBL were cultured at 10^6 /ml irradiated (5000 rad) T51 LCL. EBV-transformed lymphoblastoid cell lines (LCL), PM (Bristol-Myers Squibb Co.) and T51 (Bristol-Myers Squibb Co.) were maintained in RPMITM supplemented with 10% FBS. After 6 days, alloreactive "blasts" cells were cryopreserved. Secondary MLR were conducted by culturing thawed alloreactive blasts together with fresh irradiated T51 LCL in the presence and absence of mAbs and Ig fusion proteins. Cells were cultured in 96 well flat bottom plates (4×10^4 alloreactive blasts and 1×10^4 irradiated T51 LCL cells/well, in a volume of 0.2 ml) in RPMITM containing 10% FBS. Cellular proliferation of quadruplicate cultures was measured by uptake of [3 H]-thymidine during the last 6 hours of a 2-3 day culture. --

Please delete the paragraph at page 44, line 32, beginning "PHA-activated T cells were prepared by culturing PBLs with 1 μ g/ml PHA (Wellcome, Charlotte, NC) for five days, and one day in medium lacking PHA. Viable cells were collected by sedimentation

through Lymphocyte Separation Medium before use. Cells were stimulated with mAbs or transfected CHO cells for 4-6 hr at 37°C, collected by centrifugation and used to prepare RNA." and replace it with the following rewritten paragraph:

D14

-- PHA-activated T cells were prepared by culturing PBLs with 1 µg/ml PHA (Wellcome, Charlotte, NC) for five days, and one day in medium lacking PHA. Viable cells were collected by sedimentation through Lymphocyte Separation Medium™ before use. Cells were stimulated with mAbs or transfected CHO cells for 4-6 hr at 37°C, collected by centrifugation and used to prepare RNA. --

Please delete the paragraph at page 45, line 10, beginning "B cells were also purified from peripheral blood by panning as described by Wysocki and Sato, Proc. Natl. Acad. Sci. 75:2844 (1978), incorporated by reference herein, using anti-CD19 mAb 4G9. To measure T_h-induced Ig production, 10⁶ CD4⁺ T cells were mixed with 10⁶ CD19⁺ B cells in 1 ml of RPMI containing 10% FBS. Following culture for 6 days at 37°C, production of human IgM was measured in the culture supernatants using solid phase ELISA as described by Volkman et al., Proc. Natl. Acad. Sci. USA 78:2528 (1981), incorporated by reference herein." and replace it with the following rewritten paragraph:

D15

-- B cells were also purified from peripheral blood by panning as described by Wysocki and Sato, Proc. Natl. Acad. Sci. 75:2844 (1978), incorporated by reference herein, using anti-CD19 mAb 4G9. To measure T_h-induced Ig production, 10⁶ CD4⁺ T cells were mixed with 10⁶ CD19⁺ B cells in 1 ml of RPMI™ containing 10% FBS. Following culture for 6 days at 37°C, production of human IgM was measured in the culture supernatants using solid phase ELISA as described by Volkman et al., Proc. Natl. Acad. Sci. USA 78:2528 (1981), incorporated by reference herein. --

Please delete the paragraph at page 46, line 8, beginning "Immunoprecipitation Analysis and SDS PAGE. Cells were surface-labeled with ¹²⁵I and subjected to

immunoprecipitation analysis. Briefly, PHA-activated T cells were surface-labeled with ^{125}I using lactoperoxidase and H_2O_2 as described by Vitetta et al., J. Exp. Med. 134:242 (1971), incorporated by reference herein. SDS-PAGE chromatography was performed on linear acrylamide gradients gels with stacking gels of 5% acrylamide. Gels were stained with Coomassie Blue, destained, and photographed or dried and exposed to X ray film (Kodak XAR-5)." and replace it with the following rewritten paragraph:

D16 -- Immunoprecipitation Analysis and SDS PAGE. Cells were surface-labeled with ^{125}I and subjected to immunoprecipitation analysis. Briefly, PHA-activated T cells were surface-labeled with ^{125}I using lactoperoxidase and H_2O_2 as described by Vitetta et al., J. Exp. Med. 134:242 (1971), incorporated by reference herein. SDS-PAGE chromatography was performed on linear acrylamide gradients gels with stacking gels of 5% acrylamide. Gels were stained with Coomassie Blue, destained, and photographed or dried and exposed to X ray film (KodakTM XAR-5). --

Please delete the paragraph at page 46, line 18, beginning "Binding Assays. B7Ig was labeled with ^{125}I to a specific activity of approximately 2×10^6 cpm/pmole. Ninety-six well plastic dishes were coated for 16-24 hrs with a solution containing CTLA4Ig (0.5 μg in a volume of 0.05 ml of 10 mM Tris, pH 8). Wells were blocked with binding buffer (DMEM containing 50 mM BES (Sigma Chemical Co.), pH 6.8, 0.1% BAS, and 10% FCS) before addition of a solution (0.09 ml) containing ^{125}I B7Ig (approximately 5×10^5 cpm) in the presence or absence of competitor. Following incubation for 2-3 hrs at 23°C , wells were washed once with binding buffer, and four times with PBS. Bound radioactivity was then solubilized by addition of 0.5N NaOH, and quantified by gamma counting." and replace it with the following rewritten paragraph:

D17 -- Binding Assays. B7Ig was labeled with ^{125}I to a specific activity of approximately 2×10^6 cpm/pmole. Ninety-six well plastic dishes were coated for 16-24 hrs with a solution containing CTLA4Ig (0.5 μg in a volume of 0.05 ml of 10 mM Tris, pH 8). Wells were

D17 blocked with binding buffer (DMEMTM containing 50 mM BES (Sigma Chemical Co.), pH 6.8, 0.1% BAS, and 10% FCS) before addition of a solution (0.09 ml) containing ¹²⁵I B7Ig (approximately 5 x 10⁵ cpm) in the presence or absence of competitor. Following incubation for 2-3 hrs at 23°C, wells were washed once with binding buffer, and four times with PBS. Bound radioactivity was then solubilized by addition of 0.5N NaOH, and quantified by gamma counting. --

Please delete the paragraph at page 61, line 21, beginning "Cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 0.2mM proline, and 1μM methotrexate. COS cells were grown in DMEM supplemented with 10% FBS. CTLA4Ig was prepared in CHO cells as previously described (Example 2)." and replace it with the following rewritten paragraph:

D18 -- Cells were maintained in DMEMTM supplemented with 10% fetal bovine serum (FBS), 0.2mM proline, and 1μM methotrexate. COS cells were grown in DMEMTM supplemented with 10% FBS. CTLA4Ig was prepared in CHO cells as previously described (Example 2).

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Please delete the paragraph at page 62, line 1, beginning, "Six mutants were prepared which encoded substitutions to alanine in the highly conserved hexapeptide 98MYPPPY103 forming part of the putative CDR3-like domain (Figures 17 and 22) (Ho et al., 1989, supra.). These mutants are described in Table II." and replace it with the following rewritten paragraph:

D19 -- Six mutants were prepared which encoded substitutions to alanine in the highly conserved hexapeptide 98MYPPPY103 (SEQ ID 24) forming part of the putative CDR3-like domain (Figures 17 and 22) (Ho et al., 1989, supra.). These mutants are described in Table II. --

Please delete the paragraph at page 62, line 6, beginning, "In addition, two mutants encoding the residues P103A and Y104A (MYPPAY and MYPPPA, respectively) from the CD28Ig 99MYPPPY104 hexapeptide using CD28Ig as a template were also prepared by the same method. These mutants are also described in Table II." and replace it with the following rewritten paragraph:

D20

-- In addition, two mutants encoding the residues P103A and Y104A (MYPPAY (SEQ ID NO: 32) and MYPPPA (SEQ ID NO: 33), respectively) from the CD28Ig 99MYPPPY104 hexapeptide using CD28Ig as a template were also prepared by the same method. These mutants are also described in Table II. --

Please delete the paragraph at page 62, line 18, beginning, "These primers encoded the following sequences:

CDM8FP:5'-AATACGACTCACTATAGG

CDM8RP:5'-CACCACACTGTATTAACC"

and replace it with the following rewritten paragraph:

D21

-- These primers encoded the following sequences:

CDM8FP:5'-AATACGACTCACTATAGG (SEQ ID NO: 15)

CDM8RP:5'-CACCACACTGTATTAACC (SEQ ID NO: 16) --.

Please delete the paragraph at page 66, line 11, beginning "*Immunoprecipitation and Western blot analysis*. CTLA4/CD28Ig hybrid fusion proteins present in culture media were adsorbed to protein A-Sepharose by overnight incubation at 4°C. The beads were washed with PBS containing 0.1% Nonidet-P40 (NP40) then SDS PAGE sample buffer was added and the eluted protein was loaded onto an SDS polyacrylamide gel." and replace it with the following rewritten paragraph:

D22

-- *Immunoprecipitation and Western blot analysis.* CTLA4/CD28Ig hybrid fusion proteins present in culture media were adsorbed to protein A-SepharoseTM by overnight incubation at 4°C. The beads were washed with PBS containing 0.1% Nonidet-P40 (NP40) then SDS PAGE sample buffer was added and the eluted protein was loaded onto an SDS polyacrylamide gel. --.

Please delete the paragraph at page 77, line 9, beginning,

"CTLA4Ig MUTANT FUSION PROTEIN

AYPPPY	+++	+++	+++	-
MAPPPY	++	+	++	-
MYAPPY	+	-	+	-
MYPAPY	+++	++++++	+++	-
MYPPAY	+++	-	+	-
MYPPPA	+++	++	+++	-
AAPPPY	+	++	+++	-

CD28Ig MUTANT FUSION PROTEIN

MYPPAY	-	-	-	-
MYPPPA	-	-	-	+"

and replace it with the following rewritten paragraph: